The Role of Smo in Pancreatic cancer

Honors Research Thesis

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1. Abstract

Pancreatic Adenocarcinoma (PDAC) remains an overwhelmingly fatal disease with approximately 95% of patients dying within 5 years of diagnosis. Little clinical development has been seen despite the progress that has been made in understanding the molecular makeup of pancreatic cancer. The disease is so deadly because it metastasizes quickly and there is no effective treatment due to the dense stroma that protects the tumor because of an intense desmoplastic reaction. It has been found that the sonic hedgehog (Hh) pathway is upregulated in 70% of pancreatic adenocarcinomas. The sonic Hh ligand is overexpressed in the pancreatic adenocarcinomas and the gene coding for the ligand's receptor, Smoothened (Smo), is overexpressed in cancer associated fibroblasts. This suggests that cancer associated fibroblasts actively communicate with PDAC tumor cells and that Hh signaling plays some role in PDAC progression. To study the effect of SMO and the crosstalk between the cancer associated fibroblasts (CAFs) and the tumor, PDAC cells were grown in collected conditioned media (CM) harvested from the fibroblasts. Conditioned Media was collected from both Smo^{WT} or Smo^{null} fibroblasts. It was determined through ki67 assays and BrdU assays that when tumor cells were grown in CM form Smo^{null} cellular proliferation increased. Therefore, It was determined that the fibroblasts do communicate with the PDAC cells and when the Hh pathway was removed from the fibroblasts, PDAC proliferation increased.

2. List of Abbreviations

Bromodeoxyuridine (BrdU)

Cancer associated fibroblasts (CAF)

Conditioned media (CM)

Elas-CreER; LSL-$Kras^{G12D/+}$; $TP53^{R172H/+}$ (KPC)

Elas-CreER; LSL-$Kras^{G12D/+}$ (KC)

Hedgehog (Hh)

Mist1Kras; FspCre; Smo^{loxp} (K;C;S)

Mist1Kras; Smo^{loxp} (K;S)

Pancreatic ductal adenocarcinoma (PDAC)

Pancreatic intra-epithelial neoplasia (PanIn)

Patched (Ptch)

Platelet derived growth factor (PDGF)

Smoothened (Smo)

Transforming growth factor Beta (TGFbeta-1)

Tumor Microenvironment (TME)

3. Introduction

Pancreatic Ductal Adenocarcinoma, PDAC, is one of the most deadly cancers, with only 5% of patients surviving five years after diagnosis and a median survival of less than six months (National Cancer Institute). PDAC has such a high mortality rate because it metastasizes so quickly and there are no effective treatments available. In order to treat this cancer, a better understanding of the tumor microenvironment and how the tumor spreads within the pancreas needs to be obtained. To address this issue, Dr.Ostrowski's lab has focused on studying the tumor microenvironment and how it contributes to tumor growth.

PDAC develops in a series of steps where normal pancreatic tissue transforms into pancreatic intra-epithelial neoplasia (PanIN). A normal human pancreas is composed of acinar cells and ductal cells, which are composed of cuboidal cells with uniform nuclei. In the first stage of PDAC development, the cuboidal cells take on a more columnar phenotype, this is the characteristic of PanIN-1A lesions. PanIN-1B lesions are characterized by papillary ductal lesions and the transition to PanIN-2 lesions is marked by nuclear atypia and loss of cellular polarity. Finally, PanIN-3 lesions are characterized by a complete loss of cellular polarity and the presence of goblet cells, mucous producing cells typically found in the respiratory and digestive systems (Hingarani *et al,* 2003). Importantly, during this entire tumorigenic cascade, there is a stepwise expansion of the stroma that correlates with disease progression. By the time the tumors reach PDAC stage, they can be comprised of roughly 70% stroma (Chu *et al*, 2007).

One of the key characteristics of PDAC is the formation of a dense stroma (Mahadevan *et al*, 2007). The tumor stroma, also called the tumor microenvironment (TME), is the complex system of cells, signaling molecules, and extracellular matrix surrounding the tumor. The cells that typically surround the tumor are fibroblasts, immune cells and endothelial cells and the signaling molecules present are typically cytokines and growth factors. It has been found, that in PDAC the TME rapidly evolves during the progression of pancreatic cancer (Chu *et al*, 2007). The cancer associated fibroblasts (CAF) make up a large portion of the TME and they are thought to have a large role during PDAC development. The CAFs play a role in the development of the characteristic dense stroma of PDAC. When CAFs are activated by growth factors like TGFbeta-1 and platelet derived growth factor (PDGF), they secrete fibronectin and collagens which create a dense stroma. This dense stroma that surrounds the tumor is believed to result in decreased vascularization of PDAC which protects the tumor from chemotherapy. Additionally, CAFs are a key source of cytokines that aid the tumor-stroma crosstalk, promoting PDAC progression and metastasis (Masamune *et al*, 2009). However, the exact mechanism of the CAF and PDAC crosstalk has yet to be studied.

An important oncogene in PDAC development is the Kras gene; more than 90% of patients with pancreatic cancer have an activated Kras mutation (Hingarani *et al*, 2003). The Kras gene codes for the KRAS protein, which serves an important function in the cellular pathway that controls cellular division (US National Library of Medicine). Kras is part of the Ras family which is a signaling transducing GTPase. When mutated, they leave the protein in its GTP-bound form and therefore it constitutively interacts with the downstream effector proteins (Lee *et al*, 2010). Therefore, When Kras is

overexpressed, cellular division increases. The Kras mutant that we use in our lab is the $Kras^{G12D}$ variant, which is found in nearly all PDAC patients and is a constitutively active form of the protein. Another common mutation in human PDAC, is the inactivation of the tumor suppressor gene P53, which is found to be mutated in 70% of PDAC patients. (Kimmelmen *et al*, 2006)

Another important pathway observed to be over-activated in human PDAC is the hedgehog (Hh) signaling pathway. The Hh pathway, which plays an essential role in regulating cell proliferation and differentiation during the embryonic stage of development, is not normally expressed in adult tissue (Walter *et al,* 2010). However, it has been found that the Hh signaling pathway is re-activated in 70% of patients with PDAC (Walter *et al,* 2010). The three main Hh proteins in humans are sonic, Indian, and Desert (Walter *et al*, 2010). The ligands serve to activate the Hh pathway in neighboring cells through cellular crosstalk. In humans, the Hh ligands bind to Patched1 protein (PTCH1) on target cells, which release the suppression of smoothened (SMO), the key transducer of Hh pathway. Activated SMO relocates to the cilia membrane and initiates an intracellular down-stream signaling cascade, resulting in activation of GLI transcription factors and expression of downstream targets such as Gli1, Ptch1 and C-myc. Furthermore, it has been shown that Hh signaling promotes pancreatic tumorigenesis through a paracrine manner. However, the target cell compartments remain unclear (Ming *et al*, 1998;Katoh *et al* 2009). More evidence has indicated that Hh in CAFs is responsible for PDAC progression. When Smo inhibitors were treated on a mouse model, there was a reversal of the tight fibrotic stroma (Olive *et al*, 2009). However, recent phase II clinical trial of SMO inhibitor IPI-926 in combination with

Gemcitabine on PDAC resulted in a worse prognosis when compared to placebo groups (Infinity Corp reports, 2012). Therefore, the specific role of Hh mediated crosstalk between pancreatic tumor and stromal fibroblasts need to be elucidated to provide directions for future clinical works.

In order to study pancreatic cancer, a mouse model that mimics human PDAC development, has a similar genetic makeup, and mimics the tumor microenvironment and dense stroma of human PDAC needs to be used. Our lab uses a Mist1Kras:FspCreSmo^{loxp} (Mist1) system. The presence of a mutated Kras within Mist1, a gene expressed during pancreatic development in the acinar cells, causes the development of mixed ductal and acinar carcinomas that mimic the human condition. This mouse model, because of the fibroblast specific Cre Recombinase, allows the selective removal of Smo from fibroblasts. Thus we are able to knockout SMO specifically in the fibroblasts of a Kras-driven pancreatic tumor model to study the roles of Hh signaling during tumor formation. Preliminary results, comparing the *in vivo* microenvironment of precancerous cells with fibroblasts with Smo^{Wt} and Smo^{null}, show that there is more cellular proliferation in the ductal cells of the Smo^{null} group. Also, it was found that there was increased cellular proliferation within the fibroblasts that lacked Smo (Wu et al, unpublished). This preliminary data indicates that without Smo both the fibroblasts and precancerous ductal cells proliferate at a higher rate. My project will seek to determine if late stage tumor cells will exhibit the same increase in proliferation without Smo as the precancerous lesions. To test this, a LSL-Kras$^{G12D/+}$; TP53$^{R172H/+}$ PdxCre (KPC) mouse model, which does cause development of full PDAC in mice, will be used. In this model, a mutated Kras and a nonfunctioning p53 will be

introduced into pancreatic cells, resulting in the development of PDAC in mice. Also, I will seek to determine the effect that the CAFs alone, without the presence of other cells in the microenvironment, will have on the proliferation of PDAC cells***. The overarching hypothesis of this project is that the deletion of the hedgehog pathway in the tumor associated fibroblast promotes tumor growth.***

Specific Aims:

1. To determine if cancer associated fibroblasts are a source of tumor-stroma crosstalk.
2. To study the effect of Smo deleted fibroblasts on tumor growth through *in vitro* growth of PDAC cells with conditioned media from fibroblasts.

4. Materials and Methods

4.1 Mist1Kras; FspCre; Smo^{loxp}

A mutated Kras, $Kras^{G12D}$, is expressed in Mist1, a transcription factor that is expressed during Pancreatic development and necessary for proper pancreatic acinar organization. Smo was deleted from the fibroblasts by flanking the Smo gene with two loxp sites. These sites are recognized by FspCre, a DNA recombinase specific to fibroblast cells, which cuts the DNA at the two loxp sites and recombines the DNA. This completely removes the Smo gene from the mouse DNA. The ultimate genotype is: Mist1-Kras;FspCre;Smo^{loxp} .

4.2 KPC mouse model

The cancer cells were obtained from mice with a null tumor suppressor $p53^{R172H}$ allele and loxp sites around the regulatory stop site in front of the mutated $Kras^{G12D}$ gene. The Cre is inserted in the elastase pancreatic promoter, so it is only expressed in pancreatic cells. The cre is also placed in the elastase promoter with the human estrogen receptor, so that the Cre is only able to be induced when the synthetic ligand 4-hydroxytamoxifen is present. When Cre is active, it removes the stop site in front of the Kras gene, causing the mutant Kras to be expressed in pancreatic cells with no regulation. The loxp sites also flank a regulatory stop site in front of the null p53 allele. When the cre recombinase cuts and recombines the loxp sites, the null p53 allele is expressed. The ultimate genotype is: Elas-CreER; LSL-$Kras^{G12D/+}$; $TP53^{R172H/+}$. There are three cell lines utilized in this study; KC, KPC1, and KPC2. Both KPC cell lines were obtained from PDAC tumors have the genotype listed above. KC does not contain the mutant p53 allele but has the activated Kras giving it the ultimate genotype of Elas-

CreER; LSL-Kras$^{G12D/+}$. The KC cell line was derived PanIN-like lesions, not PDAC tumors. These cells were generously donated from Stephen F. Konieczny at Purdue University.

4.3 Genotyping

In the Smo mouse model, a polymerase chain reaction (PCR) was run to check that FspCre is present in the experimental group. PCR was also used to ensure that mutant Kras is expressed in both the control and experimental group. Also, it is needed to check for the presence of the loxp sites that flank the Smo gene. In order to isolate the genes, primers specific and antisense to the genes DNA sequence was used. In addition to primers, a buffer mix, sample DNA, dNTPs, and Taq polymerase (biolabs M0267L) were added to allow for the amplification of the desired gene.

SmoLoxP:
SmoLoxP forward: TGCGCTACAACGTGTGCCTG
SmoLoxP reverse 1: GGCGCCTACCGGTGGATGTGG
SmoLoxP reverse 2: CCGGTGGATGTGGAATGTG

For Smo+/-:
SmoForward: GCAAAGTTGGGAGTCGAG
SmoReverse: CCAAACAGCCAACTCAGC
SmoNullreverse: ATTTGTCACGTCCTGCAC

MistKras:
Mist1-Kras forward: AGGTGTCCACTAAGCACCAGT
Mist1 reverse: CTGGAAGGCATTGTTGAGTTT
Kras reverse:GCTCCAACCACCACAAGTTTA

nFspCre:
nFspCreForward: CGTGGGTTGGAGAATGTTAC
nFspCre Reverse1:ATTTCTAGTGCCCATCTCCG
nFspCre Reverse 2: CCGGTTATTCAACTTGCACC

4.4 Fibroblast Isolation

A dissected pancreas is minced in collagenase and then mixed with digestion solution (Pbs, collagenase Type II, DNase I). The tissue is then placed on a shaker at 37 degrees Celsius for 60 minutes at 225 rpm. The vial is spun down and the supernatant is removed and the tissue is plated on a 6 well plate in Dulbecco's Modified Eagle Medium (DMEM) (wu *et al*, unpublished).

The effectiveness of the isolation was tested through a Smooth Muscle Actin (SMA), Vimentin, and Yellow Fluorescent protein (YFP) stain. Both SMA and Vimentin are fibroblasts specific stains. The YFP is placed into the chromosome and then expressed in the fibroblasts alone because of an FspCre. Therefore, the presence of YFP can serve as a fibroblasts specific marker.

4.5 Conditioned Media

In order to grow cells in media with the extracellular components released by fibroblasts, the media the fibroblasts are grown in must be collected. This is called conditioned media (CM). Conditioned media from both Smo^{WT} and Smo^{null} fibroblasts was collected.

Fibroblasts were grown to 80% confluency and then washed twice with Dulbecco's Phosphate Buffered Saline (DPBS) and serum free media. Then serum free media was placed on the cells for 36 hours. After 36 hours, the media was sucked off, spun down, and filtered through a 0.2 um Thermo Scientific Nalgene Syringe Filter. The media is then stored at -20 degree Celsius. Then when the experiments are performed, fetal bovine serum (FBS) is added so that the CM is at 0.5% FBS.

4.6 Growth Curve

Each growth curve was performed on a 12 well plate, so that each count for a single day was performed three times, and those numbers were averaged to give the cell count for each day. The fibroblast growth curve was performed at passage 12, the cells were plated at 3,000 cells per well and the experiment was performed for eight days. The tumor cell growth curve was performed at passage 14, were plated at a starting concentration of 2,000 cells per well and it was performed for eight days.

4.7 Immunofluorescent Staining Protocol

All three cell lines, KC, KPC, and KPC2, were used in both the Bromodeoxyuridine (BrdU) and Ki67 stainings. Each time a staining was performed, all three cell lines were at the same passage number and the staining was performed at the same time with the same batch of conditioned media. For each type of staining, there was a baseline experiment performed in regular DMEM media with 10% FBS and 0% FBS. Then a conditioned media experiment was performed for each stain with both experimental and control conditioned media, both with 0.5% FBS. Each cell line was plated at 15,000 cells per well in regular media and 10,000 cells per well with the conditioned media. Also, each cell line was plated in four wells.

Ki67 is a cellular protein that is found on replicating cells, and therefore can serve as a marker for proliferation. The protocol for the staining performed in regular DMEM media, is as listed above and the experiment was performed when all three cell lines were at passage 23. For the conditioned media experiments, the cells were washed and

counted in FBS free media, then plated in conditioned media with 0.5% FBS. Ki67 anti-rabbit (abcam ab15580) was placed on the cells at a dilution of 1:100 for 2 hours and a fluorescent secondary was placed on the cells at a dilution of 1:25 for 1 hour. All cell lines were at passage 23 when this experiment was performed.

Each slide was photographed four times, and with four wells per cell line, there were sixteen pictures per cell line in each different condition, DMEM, control CM, and experimental CM. The pictures were taken on a fluorescent microscope and the cells positive for Ki67 were counted using Image J. The total cells positive for Ki67 were compared to the total cells, stained with DAPI, to get a percentage of positive cells to total ((100%)($Ki67^{+}$/total $DAPI^{+}$)). A ttest was performed to determine p-value.

4.8 BrdU

Bromodeoxyuridine is a synthetic nucleotide that incorporates itself into replicating genomes and therefore it can serve as a marker for replicating cells. Again with regular media cells were plated at 15,000 cells per well and each cell line had four wells. The cells were plated at passage 14. Stainings performed with CM, had cells plated at 10,000 cells per well in four wells and all three cell lines were at passage 20. DMEM staining was performed as stated above. For the CM experiments, cells were washed and counted in serum free media and plated with CM with 0.5% FBS.

BrdU was placed on the cells for 8 hours. The cells were then fixed and BrdU anti-mouse (calbiochem NA61) was placed on the cells at a concentration of 1:100 for two hours. Then a fluorescent secondary was placed on the cells for 75 minutes at a concentration of 1:75.

Each slide was photographed four times for a total of sixteen pictures per cell line per experiment. The pictures were taken on a fluorescent microscope and counted on Image J. All the BrdU positive cells were counted then compared to the total cells, ones that were positive for DAPI ((100%)($BrdU^+$/#DAPI)). A ttest was performed to determine the p-value.

4.9 Tissue Staining

Tissues were fixed in 10% neutral buffered formalin, paraffin-embedded and five micron sections prepared. They were rehydrated and stained with hematoxylin and eosin (H&E). An H&E is performed in order to view the cytoplasm and nuclei of cells. A trichrome stain was also performed on the same section of tissue. The cellular nuclei and cytoplasm and collagen was visible after a trichrome stain. Lastly, an immunohistochemistry stain was performed on the same tissue sample with Vimentin and K19. Vimentin is a fibroblast specific stain and K19 is an epithelial specific stain. The stain was performed with Vimentin anti-rabbit (cell signaling 5741S) at a concentration of 1:100 and K19 anti-rat (Troma-III-c) at a concentration of 1:200 overnight. Then a fluorescent secondary at a concentration of 1:300 was placed on the cells for an hour.

5. Results

5.1 Tumor Characterization

As previously stated, PDAC is known for having a very dense stroma. The stroma consists mainly of fibroblasts and signaling molecules such as cytokines and growth factors. In order to view the stroma, we performed H&E and Trichrome stainings of WT and Mist1Kras;FspCre;Smo$^{loxp/+}$ (KCS) pancreatic tissue were performed, the difference in microenvironment becomes very evident (Figure 1A-B). The Mist1Kras pancreatic tissue has PanIN lesions and collagen around the ducts. Collagen contributes to the characteristic dense stroma of PDAC. The WT pancreatic tissue has very few ducts, no PanIn lesions and little collagen in the pancreatic tissue.

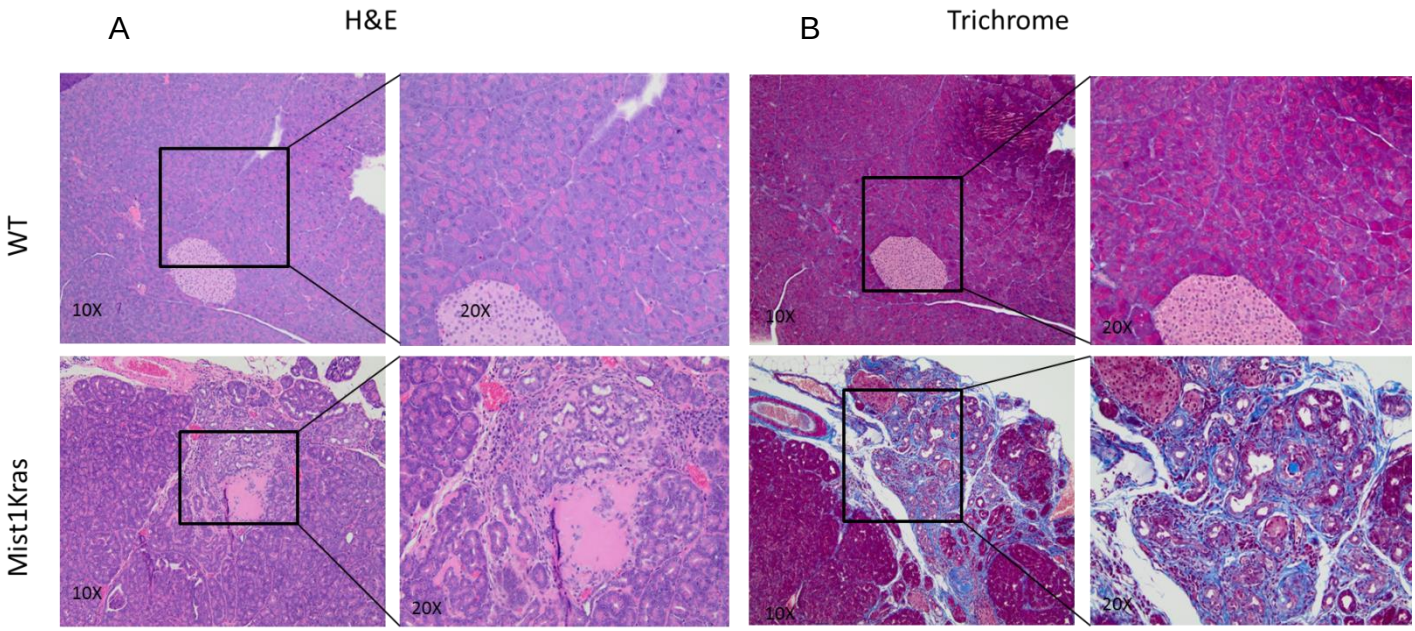


Figure 1. Mist1Kras expression results in PanIN lesions and formation of a dense stroma
A. H&E or Hematoxylin and eosin, stains for the nuclei and cytoplasm respectively. **B.** The trichrome stain allows visualization of the collagen, cytoplasm, and the nucleus.

An immunoflourescent stain was then performed in order to determine the type of cells that surrounded the ducts. The tissue was stained for the presence of fibroblasts with Vimentin (green), epithelial cells with K19 (red), and all cells with DAPI (blue) (Figure 2A-B). It was predicted that more fibroblasts would be found around the PanIn lesions in the Mist1Kras mice.

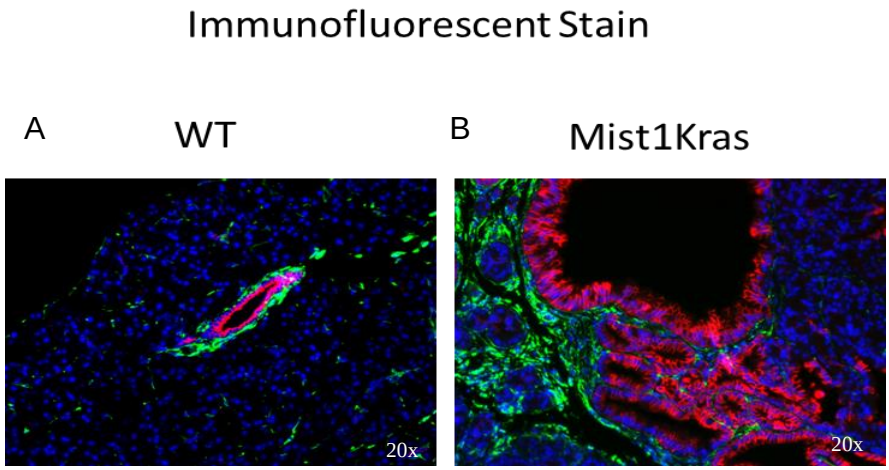


Figure 2. Mist1Kras Mouse Model has increased CAF
Cellular composition of WT and Mist1Kras Cells. Fibroblasts are visible through vimentin staining (green) and epithelial cells are visible through K19 staining (red).

As evidenced by the tissue stainings, the Mist1Kras pancreases have significantly more ducts, and have more collagen, and more fibroblasts surrounding the PanIn lesions.

5.2 Mist1Kras Mouse model

In order to study the CAFs with and without Smo, a mouse model needed to be created that could effectively delete Smo from only fibroblasts. We placed loxp sites around the Smo gene in the Smo^{null} mice and used a fibroblasts specific Cre, FspCre, to cut at the loxp sites and remove the Smo gene (Figure 3A). PCR was performed to ensure that FspCre, Kras, and loxp sites were present (Figure 3B). As predicted, in the Mist1Kras mice, *in vivo* the pancreas was inflamed and had the characteristic shape

and deformations of pancreatic cancer and the WT mice had a normal pancreas (Figure 3C).

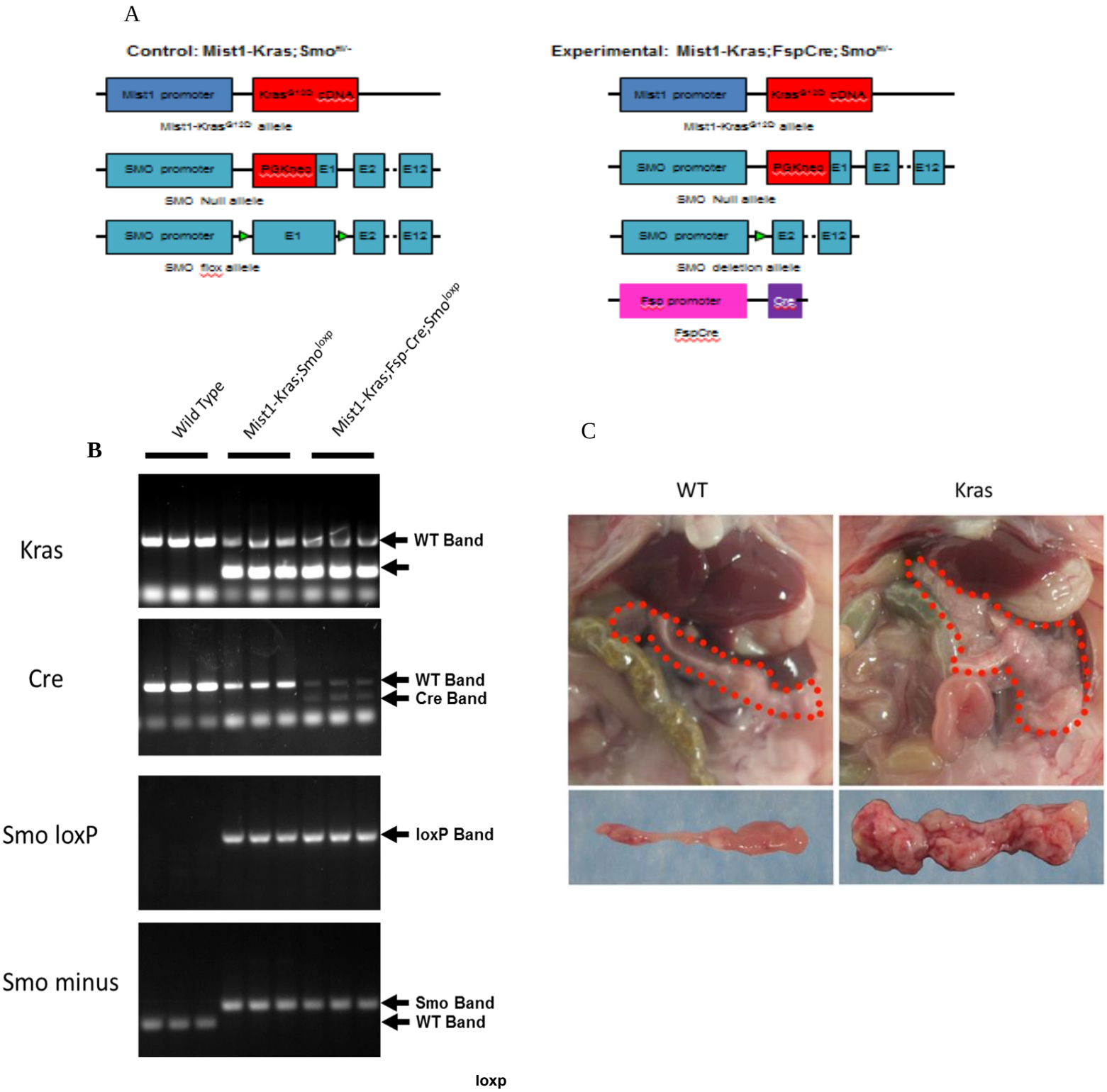


Figure 3. Mist1-Kras;Fsp-Cre;Smo genetic model.
A. Allelic representation of the mouse model. **B**.PCR was performed to confirm the presence of Fsp-Cre, Kras, Loxp, and Smo genes. **C**.Images of dissected mouse pancreases from a WT mouse and a Mist1-Kras;Fsp-Cre;Smo$^{loxp/+}$ mouse.

The growth characteristics of the Smo^{WT} and Smo^{null} *in vitro* was studied through a growth curve experiment and it was found that the absence of Smo does not affect the growth of the fibroblasts (Figure 4).

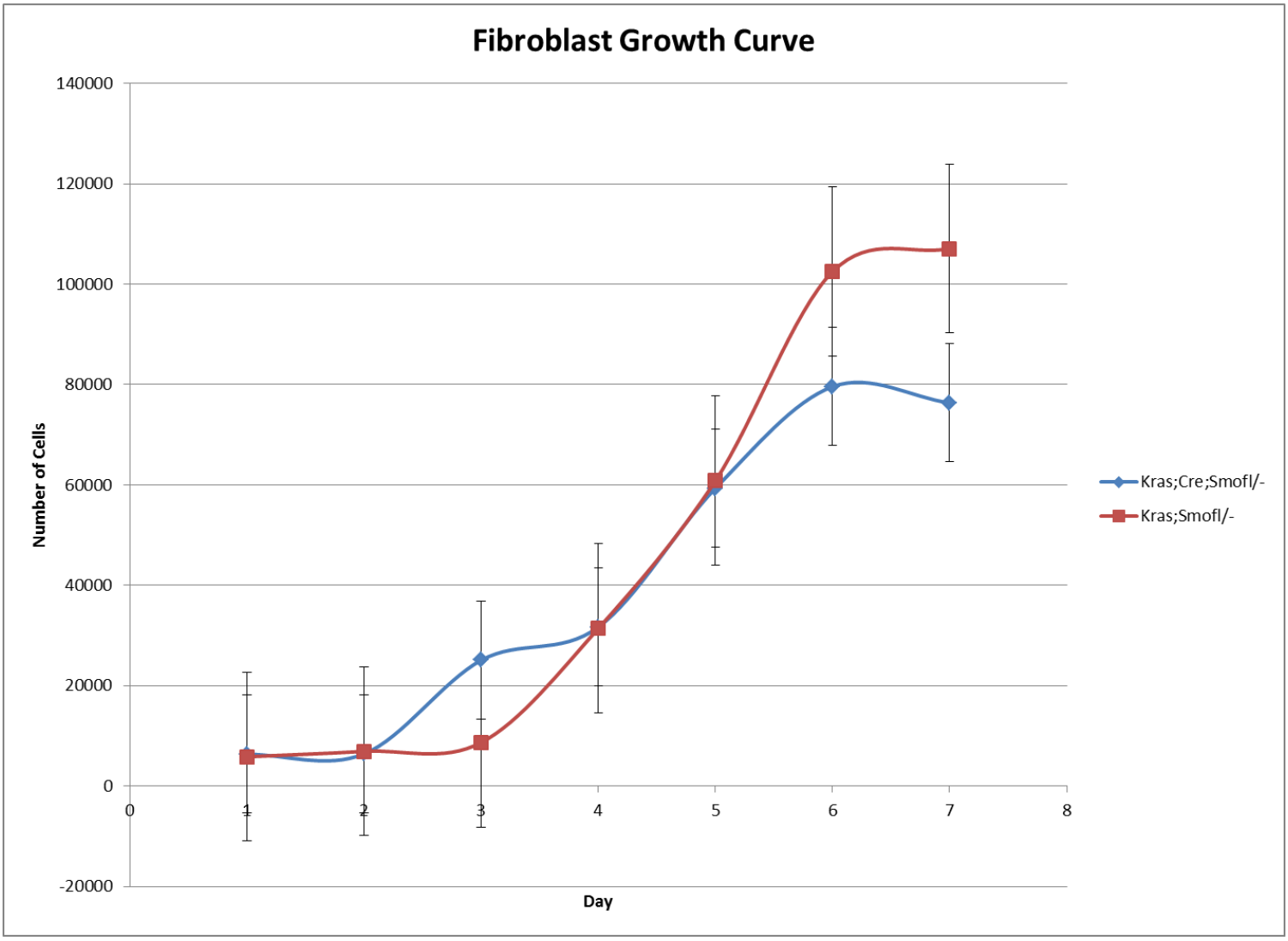
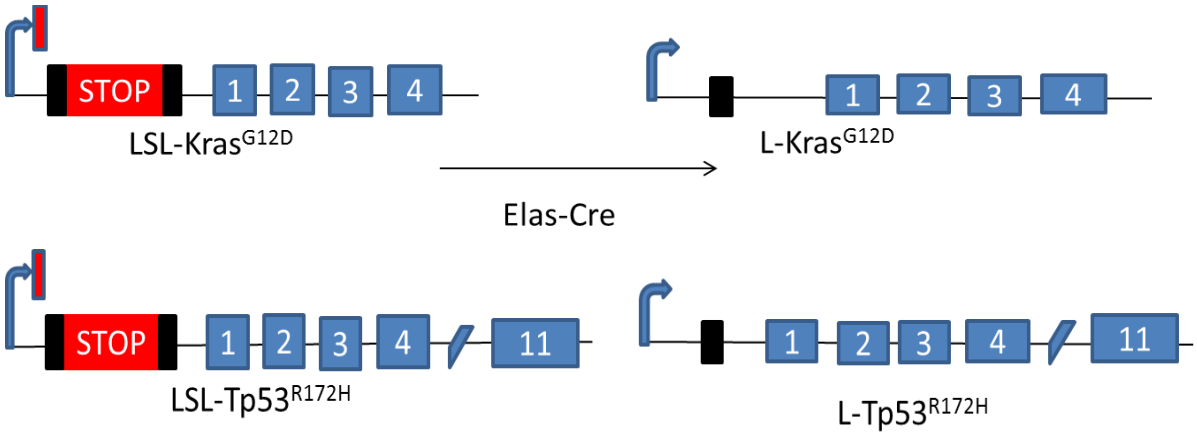


Figure4. Growth Characterization of Mist1Kras;$Smo^{loxp/+}$ and Mist1Kras;FspCre;$Smo^{loxp/+}$
A seven day growth curve experiment was performed to characterize the growth of the control and experimental fibroblasts.

5.3 KPC Mouse Model

In order to study late stage cancer cells, a different mouse model was used. This model had a mutated Kras and a non functioning P53 (Figure 5A). Two cell lines were obtained from PDAC tumors and the third was obtained from PanIn lesions. The growth rate was determined *in vitro* through a growth curve experiment. It was found that the growth of the three lines were similar (Figure 5B).

A



B

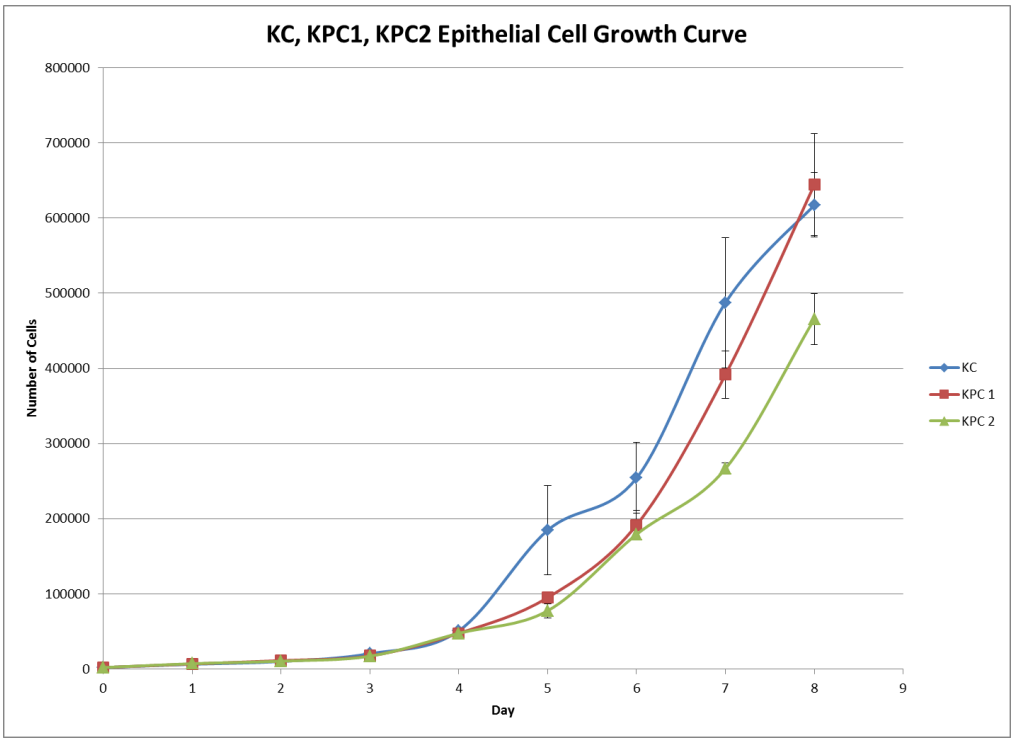
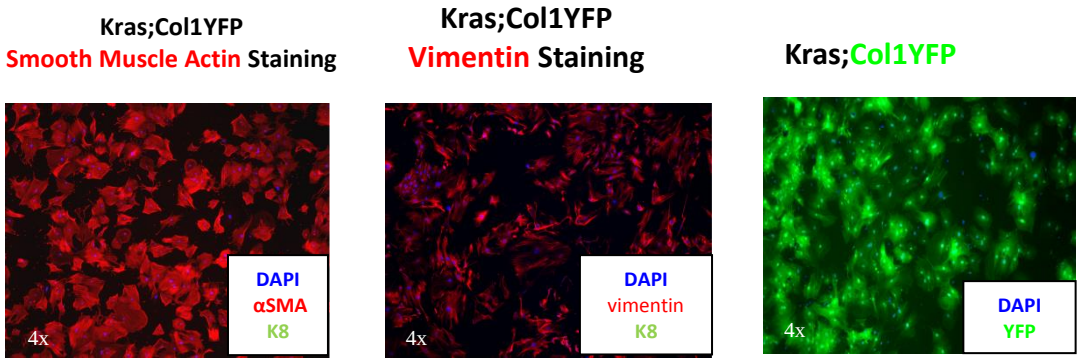


Figure 5. Elas-CreER; LSL-Kras$^{G12D/+}$; TP53 $^{R172H/+}$ Mouse Model
A Allelic representation of the PDAC genetic model. **B**. Growth Curve of the three experimental PDAC cell lines for a length of eight days.

5.4 Fibroblast

In order to determine if CAFs are the main source of tumor-stromal crosstalk, a pure fibroblasts culture will need to be isolated from the pancreas. We have optimized a technique to harvest fibroblasts that has produced cultures that are 99% pure as measured by Smooth Muscle Actin and Vimentin, both markers for fibroblasts. Also, cell stainings of our fibroblasts cultures are 0% positive for cytokeratin 8 (K8), an epithelial marker (Figure 6). To further prove that a pure fibroblasts culture was obtained, we bred a cohort of mice to have a fibroblasts specific Collagen 1 Yellow Fluorescent Protein and used the fluorescence as a way to prove that only fibroblasts existed in our culture. The culture was found to be pure based on the three stainings.

A



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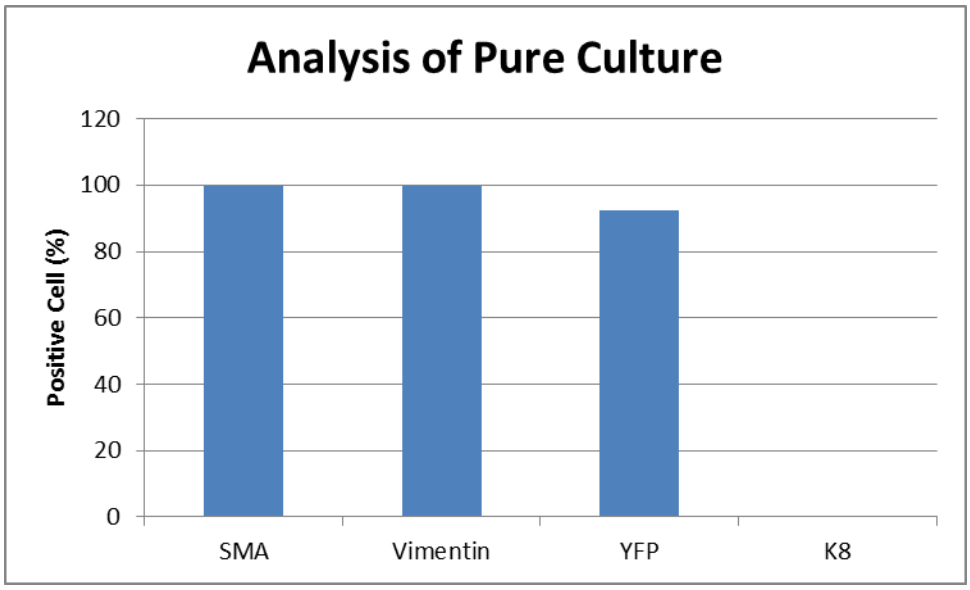


Figure 6. Confirmation of pure fibroblasts culture

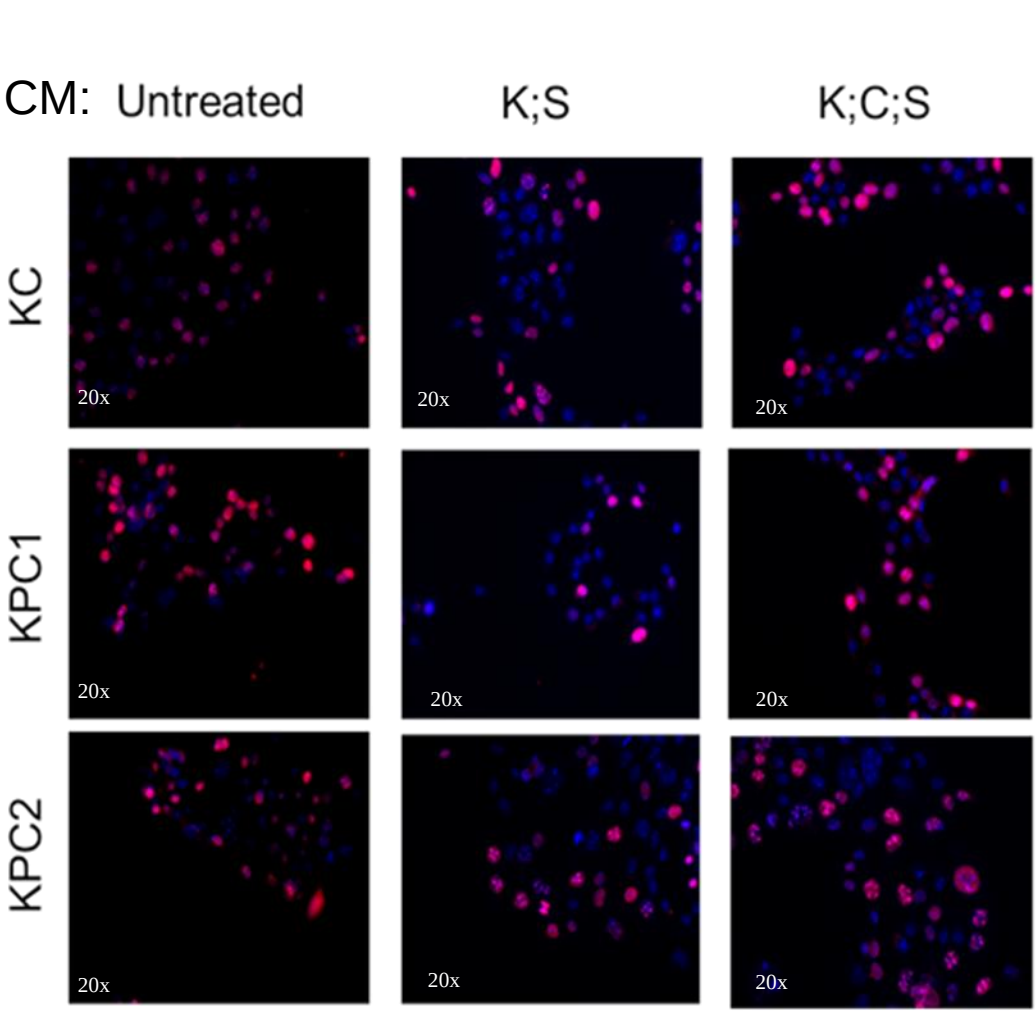
A. Images of fibroblast at 4X magnification stain for fibroblast specific markers SMA and Vimentin. The last stain is for YFP, which was genetically recombined to be expressed in fibroblasts. **B.** Quantified data of fibroblast positive cells.

5.5 Cellular Proliferation

As predicted all three cell lines, both the cells form precancerous lesions (KC) and cancerous epithelial cells (KPC1 and KPC2), had increased cellular proliferation when grown in CM from fibroblasts without Smo. Cellular proliferation rates were determined through BrdU and Ki67 stainings (Figure 7A-B; Figure 8A-B). The cells proliferation increased by about 20% when grown in CM from fibroblasts without Smo when compared to cells grown in CM from fibroblasts with Smo. When there are no Hh ligands involved in crosstalk between the tumor and stroma and therefore the Hh pathway is not activated in the CAFs, the tumor cells proliferation increased. The growth was increased so much, that when grown in CM with only 0.5% FBS, cellular proliferation was completely restored to cellular proliferation levels in regular 10% media. There was no growth associated with cells that were plated in 0% DMEM media, because the cells did not sit down on the plate, so a stain was not possible. These were results were found in both the BrdU assay and Ki67 assay.

A

B



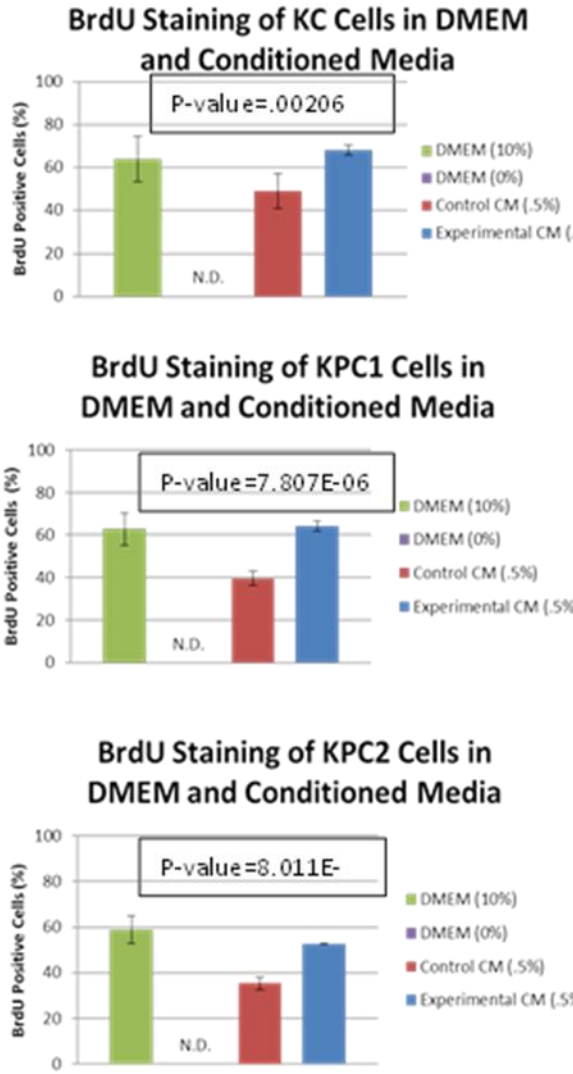
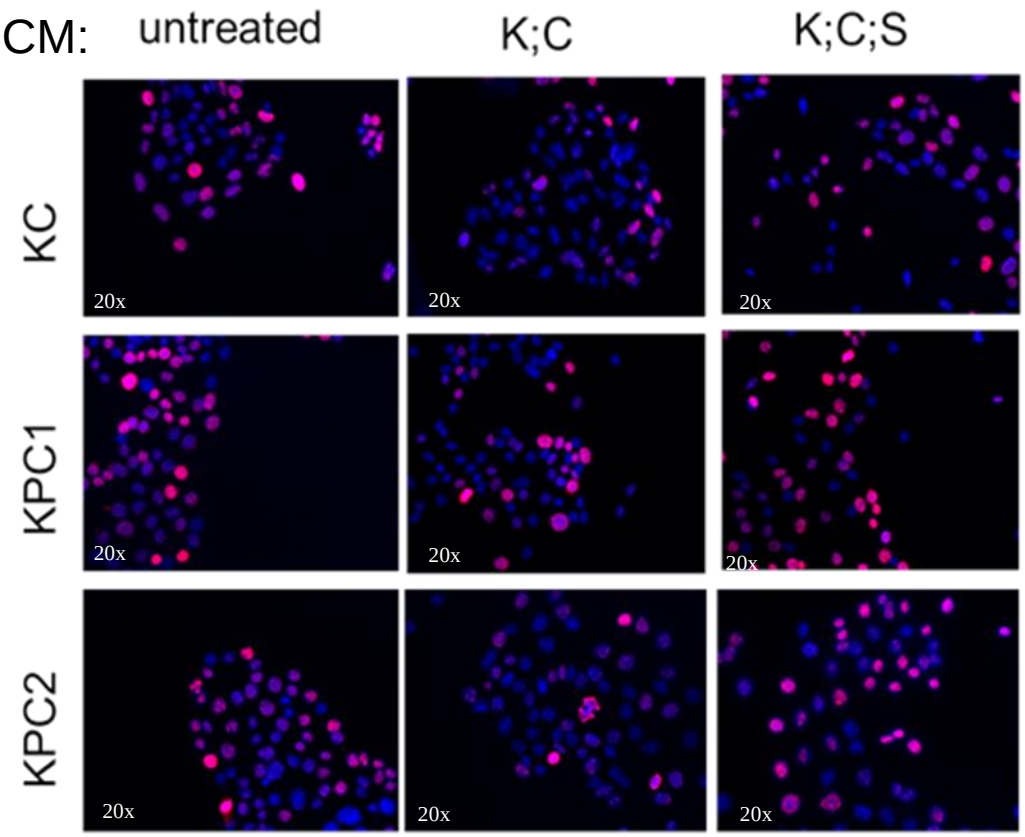


Figure 7. Conditioned Media from Smo^{null} fibroblasts increased tumor cell proliferation *in vitro*

A. Representative pictures of Cell lines KC, KPC1, and KPC2 stained for BrdU after growth in 10% DMEM, and 0.5% control and experimental CM. **B**. Qantified data of BrdU positive cells.

A



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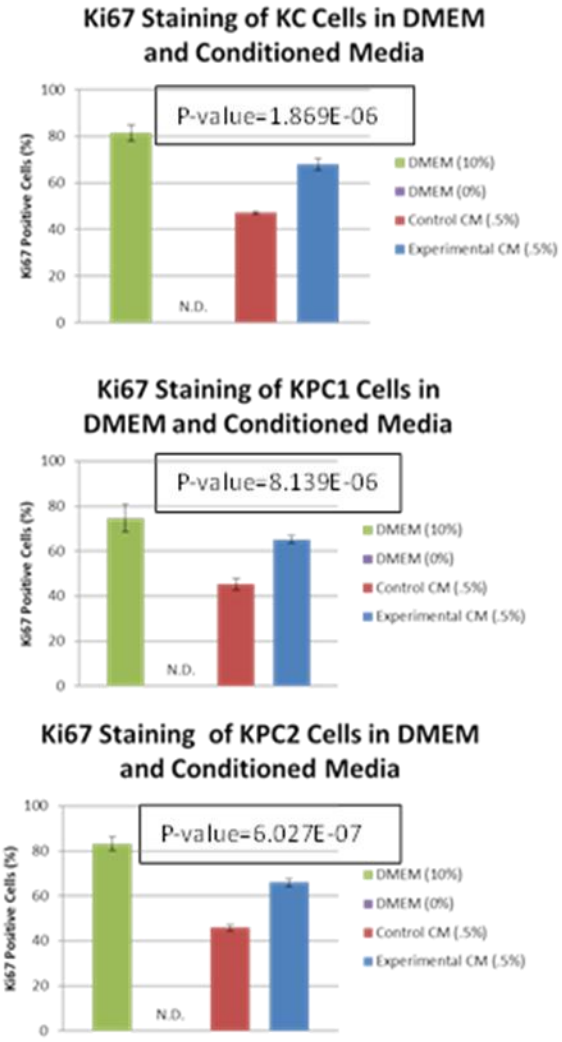

Figure 8. Conditioned Media from Smo^{null} fibroblasts increased tumor cell proliferation *in vitro*

A. Representative pictures of Cell lines KC, KPC1, and KPC2 stained for Ki67 after growth in 10% DMEM, and 0.5% control and experimental CM. **B**. Qantified data of Ki67 positive cells.

6. Discussion:

Fibroblasts were determined to play a role in the formation of the dense stroma surrounding PDAC. I*n vivo* data characterizing the pancreas of Mist1Kras mice showed that PanIn lesions were present and they had increased presence of fibroblasts and had large amounts of collagen surrounding the PanIn lesions. The collagen was produced by the CAFs and it results in a “stromal fortress” around the PDAC.

Hh Signaling, an important role in embryonic development, has shown to be important in PDAC development. *In vivo* when Smo was removed from fibroblasts around precancerous lesions, both the epithelial cells and fibroblasts had increased cellular proliferation. Smo, also has an effect *in vitro* on the growth of precancerous and cancerous epithelial cells. When both the precancerous cells (KC) and cancerous cells (KPC1, KPC2) were grown in CM from fibroblasts with Smo and without Smo, the cells in the CM with Smo had a 20% increase in cellular proliferation rates. When the Smo receptor is not present in the fibroblasts, the Hh ligand is no longer effective in the tumor-stroma crosstalk, and therefore the Hh pathway is blocked in the fibroblasts. Therefore, the Hh pathway, when present in fibroblasts, decreases cellular proliferation rates and when the Hh pathway is not present in the CAFs cellular proliferation increases. This further indicates that the fibroblasts have a role in the tumor-stroma crosstalk. Also, the deletion of Smo has the same effect on both precancerous epithelial cells and cancerous epithelial cells, because the same increase in cellular proliferation was found in both cell types.

For future studies, the *in vivo* effects of Smo deleted CAFs on cancerous epithelial cells could be studied through a co-injection study. A co-injection of

fibroblasts, both Smo^{Wt} and Smo^{null} , and cancerous mouse epithelial cells could be performed to study the effect on tumor size. Eventually the study could be performed using human cells to make it more applicable to human PDAC. Based on results presented in this manuscript, we would predict that tumor size would increase in size without the Smo present in fibroblasts.

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